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SHORT COMMUNICATION

16S rRNA profiling of bacterial communities in the brown dog tick *Rhipicephalus linnaei* from stray dogs in Perak, Malaysia

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ABSTRACT

Rhipicephalus linnaei is a widespread tick species infesting dogs and capable of transmitting pathogens of veterinary and zoonotic concern. However, its associated bacterial communities remain poorly described in Malaysia. This study profiles the bacterial microbiome of *R. linnaei* collected from stray dogs in Kampar, Perak, using 16S rRNA gene amplicon sequencing targeting the V3-V4 region. A total of 360 ticks were collected from 13 dogs, of which 290 were pooled according to life stages and sex for microbial profiling. Shannon diversity indices indicated the mixed adult/nymph pool exhibited the highest richness and evenness (H'=5.4), whereas engorged adult females displayed the lowest diversity (H'=2.35), dominated by *Gammaproteobacteria*. Principal coordinate analysis revealed distinct microbial assemblages among pools, explaining 72% of total variance. Among 137 detected genera, *Coxiella* (0.6–34%), *Staphylococcus* (0.4–29%), *Stenotrophomonas* (0.3–5%), and *Streptococcus* (0.02–6%) were consistently found across all pools. Low-abundance but clinically relevant genera, including *Ehrlichia* (0.64%) and *Nocardia* (<0.01%), were detected in adult males. The consistent presence of *Coxiella*-like endosymbionts across all stages suggests a likely symbiotic role in nutrient provisioning and reproduction. To our knowledge, this is the first 16S rRNA gene-based profiling of the bacterial communities associated with *R. linnaei* collected from stray dogs in Malaysia. This study highlights variation across pooled tick categories and contributes to improved understanding of tick-borne pathogen ecology within a One Health framework.

Keywords: *Rhipicephalus linnaei*; Tick-borne pathogen; Tick microbiome; 16S rRNA gene sequencing; Malaysia.

INTRODUCTION

Ticks are medically and veterinary important vectors capable of transmitting pathogens including bacteria, parasites, and viruses (Krupa *et al*., 2024). The brown dog tick, *Rhipicephalus linnaei,* was previously regarded as part of the *Rhipicephalus sanguineus* sensu lato complex, but recent molecular and morphological evidence has clarified it as a distinct tropical lineage that is closely associated with domestic dogs and commonly found in tropical environments (Šlapeta *et al*., 2021).

R. linnaei is known to harbour several pathogens of veterinary and public health relevance, including *Rickettsia* spp., *Ehrlichia* spp., *Coxiella burnetii*, *Borrelia* spp., and *Theileria* spp. (Moraga-Fernández *et al*., 2023). Tick-borne pathogen transmission occurs via blood feeding (Šimo *et al*., 2017), making ticks highly efficient vectors and contributing to the rising global incidence and geographical spread of tick-borne diseases.

In Malaysia, the true diversity of medically important ticks and pathogens remains under characterized, especially in domestic dogs. Previous studies have reported *Ehrlichia* spp., *Anaplasma* spp., and *Coxiella burnetii* in ticks and dogs (Koh *et al*., 2015). Stray dogs frequently carry ticks (Yan *et al*., 2024), suggesting a potential reservoir for emerging tick-borne disease risks in urban communities. However, few Malaysian studies have investigated the broader tick microbiome, which may influence pathogen acquisition, maintenance, and transmission.

16S rRNA gene amplicon sequencing enables detection of broad range of microorganisms, including novel taxa (Duron *et al*., 2017). To date, no 16S rRNA gene amplicon profiling of the microbiome of *R. linnaei* has been reported in Malaysia. In this study, we employed high-throughput 16S rRNA gene amplicon sequencing to characterise the bacterial microbiome of *R. linnaei* ticks collected from stray dogs, providing a comprehensive overview of their associated microbial diversity.

MATERIALS AND METHODS

A total of 360 dog ticks were provided by Klinik Haiwan Kampar, Perak, where they had been removed from stray dogs during routine veterinary examinations between March 2024 and May 2025. Of these, 290 ticks were included in the 16S rRNA gene sequencing analysis described in this study. The remaining 70 ticks were processed under a different analytical component of the broader study and were not included in the present analysis. Ethical approval for this study was obtained from the Universiti Malaya Institutional Animal Care and Use Committee (Approval No: T/T/22052023/13032023-02/R) and Universiti Malaya Institutional Biosafety and Biosecurity Committee (Approval No: UMIBBC/NOI/R/TNCPNI/TIDREC/007-2024-13092024), with permission granted by Klinik Haiwan Kampar for sample provision.

Ticks were preserved in 70% ethanol immediately after collection. Prior to DNA extraction, samples were surface-sterilised by sequential rinsing in 1% sodium hypochlorite (bleach), 70% ethanol, and sterile distilled water, followed by air-drying on sterile filter paper to remove external contaminants, following the established decontamination protocol described by Binetruy *et al*. (2019). The sterilised specimens were then stored at -20°C. Following surface sterilisation, each tick was individually transferred into a sterile microcentrifuge tube and mechanically homogenised as a whole specimen using a sterile disposable pestle. Genomic DNA was then extracted from each homogenate using the PrimeWay Genomic II Kit (Apical Scientific, Malaysia) according to the manufacturer's insect tissue protocol. The DNA extracts were subsequently pooled according to life stage and sex for downstream 16S rRNA gene amplicon sequencing. DNA concentration and purity were measured using a NanoQuant Infinite M200 spectrophotometer, and the DNA was stored at –20°C until downstream analysis.

Following individual DNA extraction, DNA extracts from eligible ticks were pooled according to life stage and sex, generating five composite pools designated as Dapool, Dbpool, Dcpool, Ddpool, and Dpool. Dapool comprised engorged adult females (n=24), Dbpool unengorged adult females (n=32), Dcpool adult males (n=133), and Ddpool nymphs (n=46). Dpool comprised a mixed-stage pool of adults and nymphs (n=55) collected from a single stray dog. Each pool represented combined DNA from multiple ticks within the same developmental or biological category, except for Dpool, which was analysed as a mixed-stage pool from one host.

The V3-V4 hypervariable region of bacterial 16S rRNA gene was amplified using primers 341F (5'- CCTACGGGNGGCWGCAG-3') and 805R (5'- GACTACHVGGGTATCTAATCC-3'). Amplicon libraries were prepared and sequenced on the Illumina MiSeq platform (2 x 300 bp paired-end) outsourced to AGTC Genomics Sdn. Bhd. (Kuala Lumpur, Malaysia). Raw reads were processed using CLC Genomics Workbench v24 (Qiagen, Germany). Quality trimming was performed using default parameters, with a quality limit of 0.05 and ambiguous nucleotides exceeding two bases were trimmed. Automatic adapter removal was enabled, and trimming was applied from the 3' end of each read. Both forward and reverse reads were processed. Operational taxonomic units (OTUs) were clustered at 97% similarity, and taxonomic assignment was performed against Greengenes v13.8 reference database. Default parameters were applied, allowing the creation of new OTUs for sequences not represented in the reference set, with a taxonomy similarity threshold of 97% and a minimum occurrence of 2. Alignment scoring parameters included a k-mer size of 6, mismatch cost=1, gap cost=4, chimaera crossover cost=3, maximum unaligned end mismatches=5, and minimum score=40. Chimaera detection was performed using the UCHIME algorithm, and an abundance table and OTU sequence list were generated. Alpha-diversity (Shannon) and beta-diversity (Bray-Curtis dissimilarity, principal coordinates analysis; PCoA) were computed to assess bacterial diversity across pools.

RESULTS

A total of 290 dog ticks (*R. linnaei*) were processed and separated into different pools according to their respective tick life stage and sex (Table 1). Dcpool had the highest total number (133 ticks) and Dapool had the lowest (24 ticks); the highest total read count from Dcpool (331 330 reads) and the lowest was Dpool (226 184 reads).

According to Figure 1, the nymph pool (Ddpool) exhibited the highest class-level evenness of bacterial communities, showing a relative abundance of *Gammaproteobacteria* comparable to that observed in the mixed-stage pool (Dpool). In contrast, the engorged adult female pool (Dapool) exhibited the lowest bacterial class diversity but showed the highest relative abundance of *Gammaproteobacteria* among all pools. *Gammaproteobacteria, Bacilli* and *Clostridia* were consistently detected across every pool.

Table 1. Overview of the dog ticks (*R. linnaei*) pooled samples. The samples were pooled according to their life stages & sex; number of tick individuals pooled, and the total reads count of each pool are also shown

Tick Pool Code	Tick Life Stages	Number of Ticks	Total Reads Count
Dpool	Adult/Nymph	55	226 184
Dapool	Engorged, Adult Female	24	311 232
Dbpool	Unengorged, Adult Female	32	326 926
Dcpool	Adult Male	133	331 330
Ddpool	Nymph	46	305 086
Total		**290**	**1 500 758**

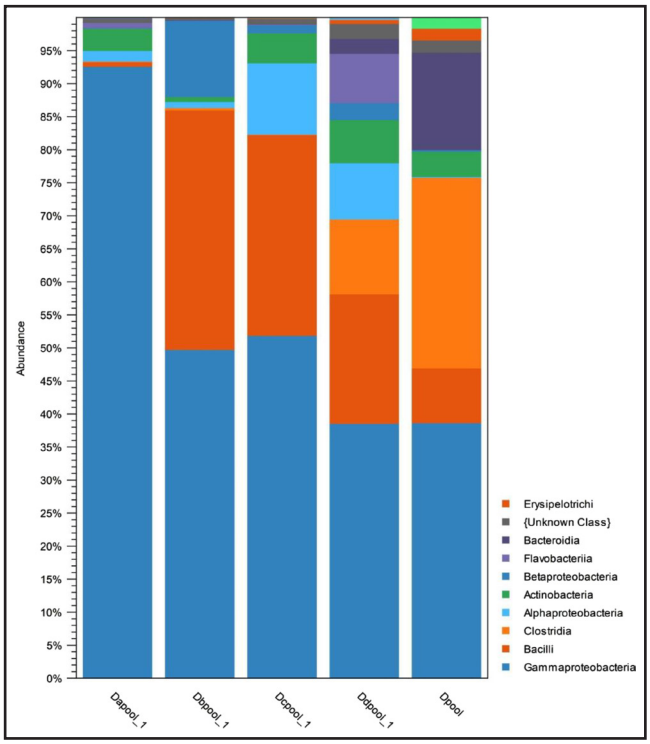

Figure 1. Relative abundance of bacterial populations at the class level in pooled dog tick (*R. linnaei*) samples according to their respective life stages and sex.

Shannon diversity indices (H') were used to evaluate the richness and evenness of bacterial communities across the pooled *R. linnaei* samples (Figure 2). The mixed adult/nymph pool (Dpool) exhibited the highest diversity (H'=5.4), indicating a more even distribution of bacterial taxa, whereas the engorged adult female pool (Dapool) displayed the lowest diversity (H'=2.35), dominated largely by *Gammaproteobacteria*. The remaining pools showed intermediate diversity values, suggesting moderate variation in microbial composition across developmental stages and sexes. Total sequencing reads per pool ranged from 226,184 to 331,330 (Table 1), confirming sufficient coverage for reliable community profiling. These results indicate that feeding status and life stage may influence microbial richness and evenness within *R. linnaei* populations.

Beta diversity analysis using Principal Coordinates Analysis (PCoA) based on Bray-Curtis dissimilarity revealed distinct bacterial community structures among the pooled *R. linnaei* samples (Figure 3). The first two principal coordinates explained 39.43% and 32.83% of the total variance, respectively, accounting for 72.26% of overall community variation. The pools were dispersed across the plot with no tight clustering, indicating marked compositional differences among life stages and sexes. The engorged adult female pool (Dapool) exhibited the greatest separation from the other pools, while the mixed-stage (Dpool), unengorged adult female (Dbpool), and nymph (Ddpool) pools grouped more closely, suggesting partial overlap in their bacterial assemblages. The adult male pool (Dcpool) occupied an intermediate position between these groups.

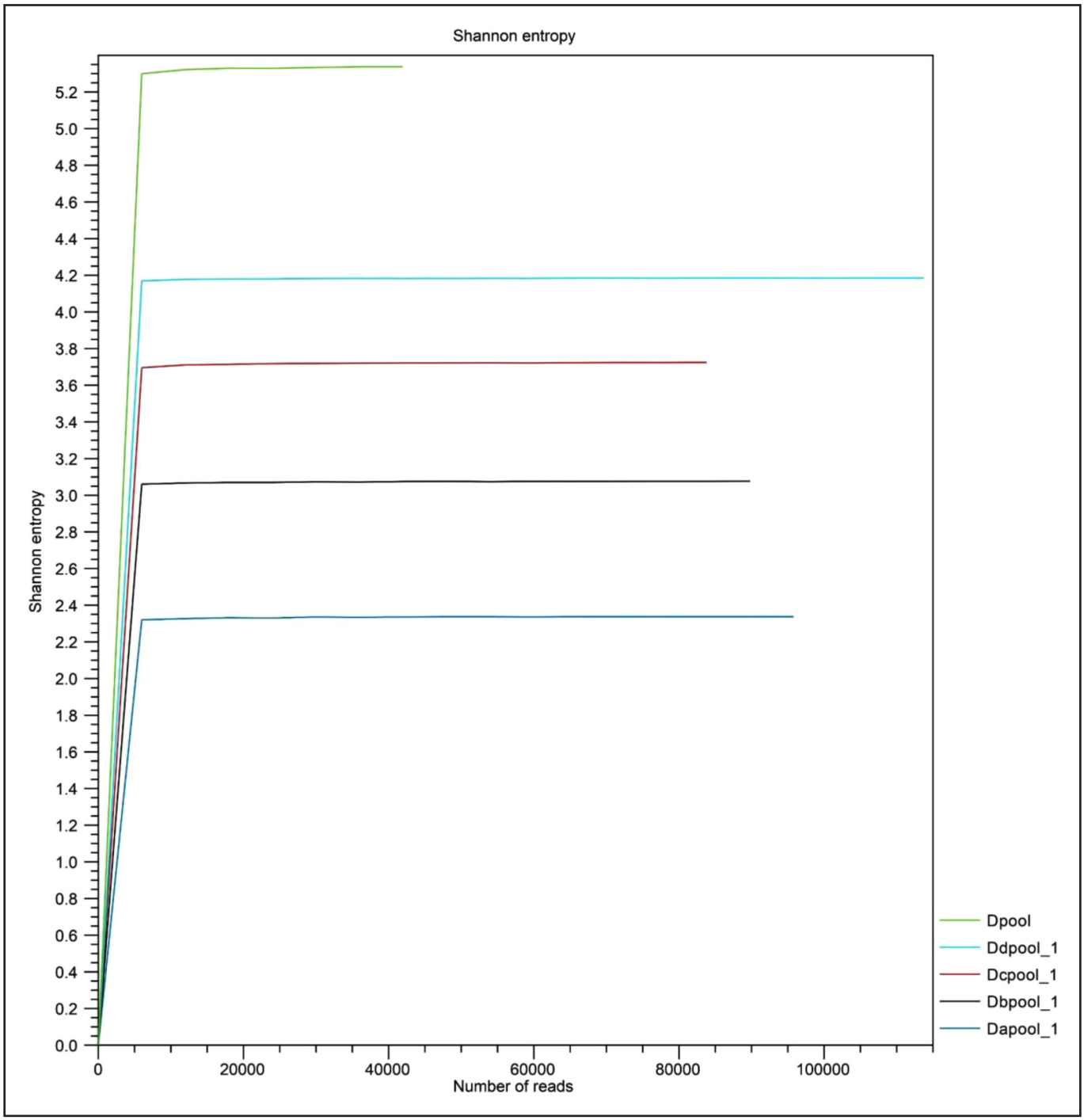

Figure 2. Line graph of Shannon entropy for each of the pooled dog tick (*R. linnaei*) samples against their respective number of reads. It measures both species' richness (number of different species or taxa) and evenness (the relative abundance of each species).

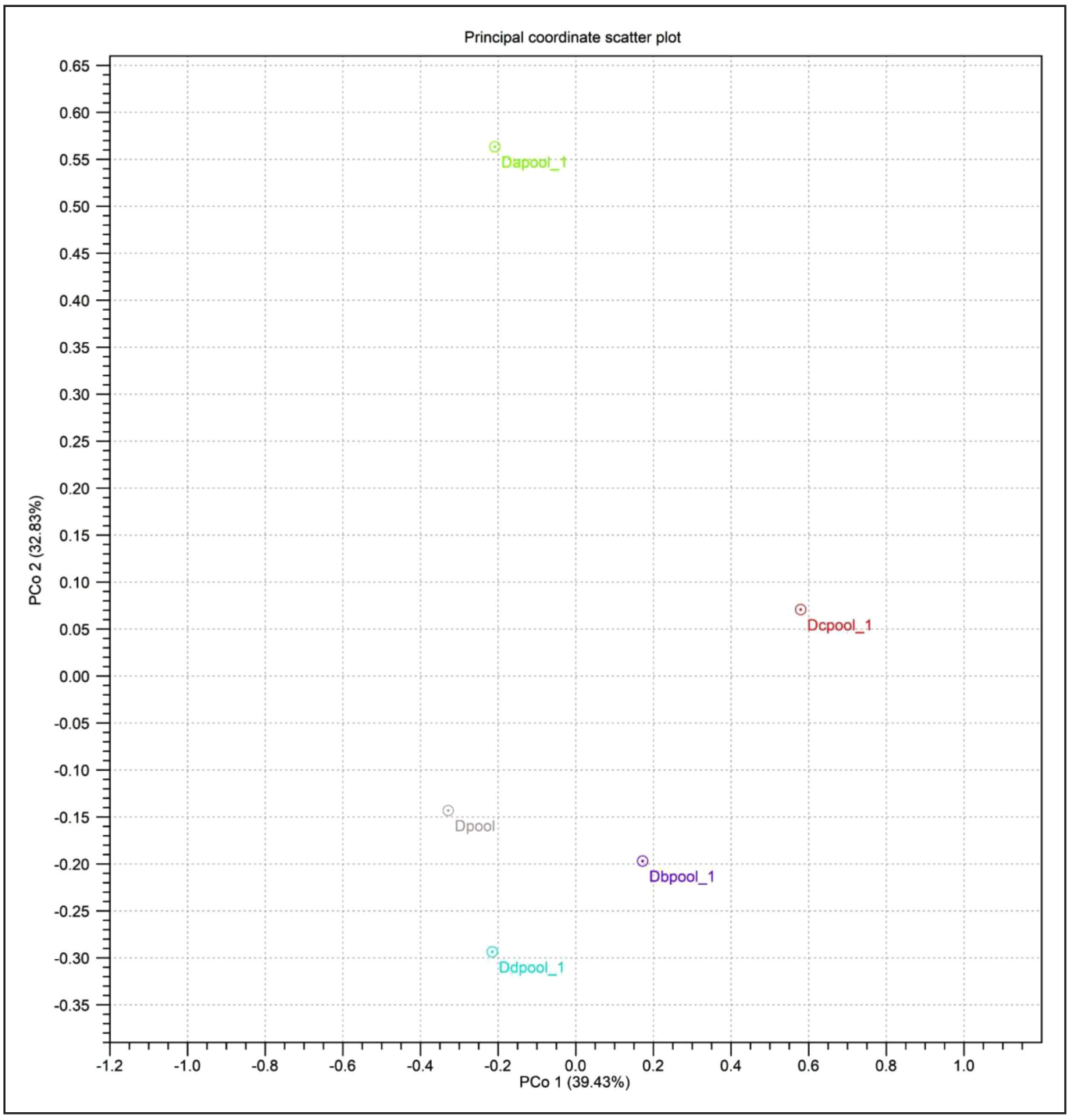

Figure 3. Principal Coordinates Analysis (PCoA) plot based on Bray–Curtis dissimilarity of bacterial communities from pooled dog tick (*R. linnaei*) samples. Each point represents a pooled sample that is indicated by different colours (gray = Adult/Nymph, green = Engorged, Adult Female, purple = Unengorged, Adult Female, red = Adult Male, teal = Nymph). The x-axis (PCo 1) explains 39.43% of the variation, while the y-axis (PCo 2) explains 32.83%. Bray–Curtis's dissimilarity measures differences in microbial community composition by incorporating the relative abundance of taxa.

A total of 137 bacterial genera were detected across the pooled *R. linnaei* samples. The top 20 genera are presented in Table 2, where *Coxiella* (0.60–34%), *Staphylococcus* (0.38–29%), *Stenotrophomonas* (0.29–5%), and *Streptococcus* (0.02–6%) were consistently identified in all pools. Overall, 42 genera of interest were classified into five major categories (Table 3): two clinically important, six putative endosymbionts, twenty-two potentially pathogenic (with unknown transmission status in ticks), eight environmental, and four mammalian commensal genera. The two clinically important genera, *Ehrlichia* (0.64%) and *Nocardia* (<0.01%), were each detected only in the adult male pool (Dcpool) at low relative abundance. Endosymbiotic and commensal genera were the most consistently represented across all pools, whereas several potentially pathogenic taxa, such as *Succinivibrio*, *Deinococcus*, *Mycobacterium*, *Neisseria*, *Flavobacterium*, and *Pasteurella*, occurred at very low abundance (<0.36%).

Table 2. Relative abundance of top 20 genera detected across the five pooled dog tick samples based on 16S rRNA gene sequencing. The relative abundance values are presented in percentage (%)

Bacterial Genus	Relative abundance (%)				
	Dapool	Dbpool	Dcpool	Ddpool	Dpool
Coxiella	**9.00**	**19.00**	0.60	**33.00**	**34.00**
Proteus	0.00	**29.00**	**44.00**	0.00	0.00
Staphylococcus	0.38	**22.00**	**29.00**	**18.00**	1.00
Vagococcus	0.25	**13.00**	0.82	0.00	0.00
Bacteroides	0.02	0.09	0.00	1.00	**9.00**
Oligella	0.01	**8.00**	0.44	0.00	0.00
Corynebacterium	**3.00**	0.47	**3.00**	0.00	0.85
Streptococcus	0.02	0.02	0.02	1.00	**6.00**
Blautia	0.02	0.03	<0.01	**3.00**	**4.00**
Elizabethkingia	0.02	0.00	<0.01	**7.00**	0.00
Stenotrophomonas	0.82	0.37	0.31	**5.00**	0.29
Rhodococcus	0.01	0.05	0.50	**5.00**	0.00
Prevotella	0.00	0.01	<0.01	0.95	**4.00**
Providencia	0.00	1.00	**3.00**	0.00	0.00
Faecalibacterium	0.02	0.04	0.00	0.47	**3.00**
Bifidobacterium	0.02	0.02	<0.01	0.35	**3.00**
Ruminococcus	0.00	0.01	<0.01	1.00	**2.00**
Acinetobacter	**2.00**	0.13	0.49	0.00	0.13
Kerstersia	0.00	0.04	0.01	**2.00**	0.00
Klebsiella	**2.00**	0.00	0.02	0.00	0.02

Footnote: Values of >1% are highlighted in bold.

DISCUSSION

This study provided the first 16S rRNA gene-based profiling of the bacterial communities associated with *R. linnaei* collected from stray dogs in Malaysia. The analysis revealed diverse bacterial assemblages varying across life stages and sexes, indicating that physiological status and developmental stage influence microbial composition. The sequencing generated between 226,184 and 331,330 reads per pool, providing adequate coverage for community characterisation.

The alpha diversity analysis showed that the mixed adult/nymph pool (Dpool) exhibited the highest bacterial richness and evenness (H'=5.4), suggesting exposure to a broader range of environmental and host-derived microorganisms. In contrast, the engorged adult female pool (Dapool) displayed the lowest diversity (H'=2.35), dominated by *Gammaproteobacteria*. This pattern agrees with previous findings that blood-feeding can restructure and reduce microbial diversity in engorged ticks (Jiang *et al*., 2024).

The beta diversity assessment (PCoA) based on Bray-Curtis dissimilarity explained 72.26% of total variance (PCo1 = 39.43%, PCo2 = 32.83%) and demonstrated clear separation of microbial communities among pools. The engorged female pool (Dapool) was most distinct, while the unengorged female (Dbpool), nymph (Ddpool), and mixed-stage (Dpool) pools clustered more closely, suggesting partial overlap in their bacterial assemblages. The adult male pool (Dcpool) occupied an intermediate position between these groups, indicating a distinct but partially overlapping bacterial composition relative to the other pooled categories.

The high diversity observed in Dpool may reflect its mixed-stage composition, as the inclusion of both adults and nymphs could have combined bacterial taxa associated with different developmental stages and increased overall richness and evenness. Nevertheless, because Dpool originated from a single host, it should be interpreted as an additional descriptive mixed-stage sample rather than as directly comparable to the category-based composite pools. The uneven distribution of certain genera, such as *Proteus*, may similarly reflect stage-related or host-associated variation, although non-detection in some pools should be interpreted cautiously because pooled 16S sequencing provides relative abundance rather than confirmed absence. The exclusive low-abundance detection of *Ehrlichia* in Dcpool may represent limited carriage within a subset of adult male ticks, but this observation requires confirmation by targeted pathogen-specific PCR assays, preferably on individual tick samples. Likewise, the absence of *Anaplasma* in the present dataset, despite previous reports in Malaysia, may be attributable to differences in geography, host population, pathogen prevalence, and the lower sensitivity of broad-range 16S amplicon sequencing compared with pathogen-specific PCR assays.

Across all pools, *Coxiella*, *Staphylococcus*, *Stenotrophomonas*, and *Streptococcus* were consistently abundant. The dominance of *Coxiella*-like sequences (0.6–34%) in every pool supports its role as a primary endosymbiont in *Rhipicephalus* ticks, likely contributing to essential physiological functions such as vitamin synthesis and reproductive fitness (Cibichakravarthy *et al*., 2022; Zeng *et al*., 2022). Low-abundance detection of *Ehrlichia* (0.64%) and *Nocardia* (<0.01%) in adult males is noteworthy, as *Ehrlichia canis* is a well-known canine pathogen transmitted by *R. sanguineus* sensu lato in Malaysia (Koh *et al*., 2015). Although these genera occurred at low relative abundance, their presence underscores the potential of *R. linnaei* as a carrier of clinically relevant bacteria.

Several other genera detected, including *Proteus*, *Vagococcus*, *Corynebacterium*, and *Bacteroides*, are commonly associated with host skin or gut microbiota, suggesting transient acquisition through blood-feeding or contact with the host's surface. The occurrence of *Proteus mirabilis*, a known zoonotic opportunist, may indicate potential veterinary or public health implications (Liu *et al*., 2025). In contrast, environmental genera such as *Acinetobacter* and *Stenotrophomonas* likely reflect habitat-related exposure.

Table 3. Relative abundance of significant bacterial genera detected across the five pooled dog tick samples based on 16S rRNA gene sequencing. The relative abundance values are presented in percentage (%)

Bacterial Genus	Relative Abundance (%)					Number of positive pooled samples
	Dapool	Dbpool	Dcpool	Ddpool	Dpool	
Clinically important genera						
Ehrlichia	0.00	0.00	0.64	0.00	0.00	1/5
Nocardia	0.00	0.00	<0.01	0.00	0.00	1/5
Putative endosymbiont genera						
Coxiella	**9.00**	**19.00**	0.60	**33.00**	**34.00**	5/5
Collinsella	0.01	0.00	0.00	0.00	1.00	2/5
Hyphomicrobium	0.00	0.00	0.59	0.33	0.00	2/5
Peptostreptococcus	0.00	0.00	<0.01	0.66	0.00	2/5
Delftia	<0.01	0.00	<0.01	0.62	<0.01	4/5
Enhydrobacter	0.36	0.04	0.07	0.00	0.02	4/5
Potentially pathogenic (tick transmission unknown)						
Proteus	0.00	**29.00**	**44.00**	0.00	0.00	2/5
Staphylococcus	0.38	**22.00**	**29.00**	**18.00**	1.00	5/5
Vagococcus	0.25	**13.00**	0.82	0.00	0.00	3/5
Bacteroides	0.02	0.09	0.00	1.00	**9.00**	4/5
Oligella	0.01	**8.00**	0.44	0.00	0.00	3/5
Corynebacterium	**3.00**	0.47	**3.00**	0.00	0.85	4/5
Streptococcus	0.02	0.02	0.02	1.00	**6.00**	5/5
Elizabethkingia	0.02	0.00	<0.01	**7.00**	0.00	2/5
Stenotrophomonas	0.82	0.37	0.31	**5.00**	0.29	5/5
Rhodococcus	0.01	0.05	0.50	**5.00**	0.00	4/5
Prevotella	0.00	0.01	<0.01	0.95	**4.00**	4/5
Providencia	0.00	1.00	**3.00**	0.00	0.00	2/5
Bifidobacterium	0.02	0.02	<0.01	0.35	**3.00**	5/5
Acinetobacter	**2.00**	0.13	0.49	0.00	0.13	4/5
Kerstersia	0.00	0.04	0.01	**2.00**	0.00	3/5
Klebsiella	**2.00**	0.00	0.02	0.00	0.02	3/5
Succinivibrio	0.00	0.00	0.00	0.00	0.36	1/5
Deinococcus	0.01	0.00	0.00	0.30	0.00	2/5
Mycobacterium	0.00	0.08	0.00	0.08	0.00	2/5
Neisseria	0.00	0.01	0.00	0.00	0.00	1/5
Flavobacterium	0.00	0.00	0.02	0.00	0.00	1/5
Pasteurella	0.00	0.00	0.00	0.05	0.00	1/5
Environmental bacteria						
Bacillus	0.00	<0.01	0.25	0.72	0.00	3/5
Luteimonas	0.47	0.00	0.00	0.00	0.00	1/5
Mesorhizobium	<0.01	0.04	0.00	0.39	0.00	3/5
Devosia	0.00	<0.01	0.05	0.19	0.00	3/5
Sphingomonas	0.01	0.01	0.21	0.00	0.00	3/5
Methylobacterium	0.01	0.00	0.00	0.15	0.00	2/5
Agromyces	0.02	0.02	0.01	0.00	0.00	3/5
Nitrobacter	0.00	0.00	0.03	0.00	0.00	1/5
Mammalian commensal genera						
Blautia	0.02	0.03	<0.01	**3.00**	**4.00**	5/5
Faecalibacterium	0.02	0.04	0.00	0.47	**3.00**	4/5
Ruminococcus	0.00	0.01	<0.01	1.00	**2.00**	4/5
Coprococcus	0.00	0.00	0.00	0.07	**2.00**	2/5

Footnote: Values of >1% are highlighted in bold.

Collectively, these findings indicate that *R. linnaei* harbours a complex bacterial community influenced by developmental stage and feeding condition. While the detection of potential pathogens suggests a need for targeted surveillance, the consistent presence of *Coxiella*-like endosymbionts highlights their likely symbiotic importance in tick physiology. This preliminary work establishes a baseline microbial profile for *R. linnaei* in Malaysia and contributes to a broader understanding of tick-microbe-host interactions within a One Health framework. Future studies incorporating individual-level sequencing, quantitative PCR validation, and host-pathogen correlation analyses will further clarify the ecological and epidemiological significance of these associations.

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Conflict of Interests

The authors declare no conflict of interests.

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